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10.1681/ASN.2016040387

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MAGI2 mutations are responsible for Congenital Nephrotic Syndrome.

Agnieszka Bierzynska[1], Katrina Soderquest[2], Philip Dean[3], Elizabeth Colby[1], Ruth Rollason[1], Caroline Jones[4], Carol D. Inward[1], Hugh J. McCarthy[1], Michael A. Simpson[5], Graham M Lord[2], Maggie Williams[3], Gavin I. Welsh[1], Ania B. Koziell*[2], Moin A. Saleem*[1] on behalf of RADAR the UK SRNS Study Group

[1]Bristol Renal and Children's Renal Unit, School of Clinical Sciences, University of Bristol, United Kingdom

[2]Division of Transplantation Immunology and Mucosal Biology, Department of Experimental Immunobiology, Faculty of Life Sciences and Medicine, King's College London, London, UK

[3]Bristol Genetics Laboratory, North Bristol NHS Trust, United Kingdom

[4]Alder Hey Children's Hospital, Liverpool, United Kingdom

[5]Division of Genetics and Molecular Medicine, Faculty of Life Sciences and Medicine, King's College London, London, UK

*joint senior authors

Abstract

Steroid-resistant nephrotic syndrome (SRNS), a heterogeneous disorder of the renal glomerular filtration barrier results in impairment of glomerular permselectivity. Inheritance of genetic SRNS may be autosomal dominant or recessive, with a subset of autosomal recessive SRNS presenting as congenital nephrotic syndrome (CNS). Currently mutations in 53 genes are associated with human SRNS yet these explain no more than 30% of hereditary and only 20% of sporadic cases. The encoded proteins are expressed in podocytes and malfunction leads to a universal end point of podocyte injury, glomerular filtration barrier disruption and SRNS. Although *MAGI2,* (Membrane Associated Guanylate Kinase, WW And PDZ Domain Containing 2) was recently shown to interact with nephrin and regulate the podocyte cytoskeleton and slit diaphragm dynamics, *MAGI2* mutations have not been previously described in human SRNS. Here, we identify novel disease causing mutations in *MAGI2* through whole exome sequencing of a deeply phenotyped cohort of childhood onset SRNS, resulting in congenital onset SRNS. Two unique frameshift mutations and one duplication were detected in three patients (2 families), two siblings shared the same homozygous frameshift mutation whereas one sporadic case exhibited compound heterozygosity. Two mutations were predicted to introduce premature stop codons and one to result in read-through of the normal translational termination codon. Mutations resulted in lack or diminished expression of podocyte MAGI2 expression. Our data supports the novel finding that mutations in the *MAGI2* gene are causal for congenital SRNS.

Introduction

Steroid-resistant nephrotic syndrome (SRNS), a disorder of glomerular filtration results in severe proteinuria, hypoalbuminaemia and oedema. Currently, 53 genes are implicated (Table S1) but these account for only 20–30% of hereditary and only 10–20% of sporadic cases supporting significant genetic heterogeneity. [1-8] To date, all known SRNS-associated genes encode proteins expressed in podocytes (or associated basement membrane), polarised cells connected by highly specialised junctions called slit diaphragms. Correct podocyte morphology is essential for maintaining GFB integrity and the podocyte actin cytoskeleton is tightly regulated by cell surface receptors including the multi-protein complex at the slit diaphragm. Known SRNS gene mutations disrupt key cellular functions resulting in podocyte injury and disruption of glomerular permselectivity. Typically, patients with a mutation in an SRNS-associated gene are less likely to respond to immunosuppressive treatment, but have a reduced risk of disease recurrence post kidney transplant.[9] SRNS may present at birth (Congenital Nephrotic Syndrome, CNS), usually with an early and severe phenotype with about 80%[10] of cases ascribed to only 6 causal genes: *NPHS1*, *NPHS2*, *LAMB2*, *WT1*, *PLCE1*, and *LMX1B*[11], all key players in podocyte biology – the genetic cause of the remainder is unknown. The majority of SRNS cases in childhood have an autosomal recessive mode of inheritance.

Results

Screening the known nephrotic genes

We expanded the study originally described in McCarthy *et al*[1] and performed whole exome sequencing on a deeply phenotyped cohort of 187 childhood SRNS cases (onset < 18 years, 11.8% familial, 7% consanguineous, 48.7% female, 51.3% male, 69.5% Caucasian, 30.5% south Asian, mixed race, African and East Asian) collected via a UK-wide Registry.

First, the cohort was screened for presence of disease causing mutations in the 53 published genes known to be associated with SRNS (Table S1; mapping statistics are presented in Table S2). Our findings correlated with previous published studies[2, 12] in that mutations in known SRNS genes were only detected in approximately 25% of cases. Assuming that mutations in the exome were present in a proportion of the remaining 75% of cases, variants detected in the whole exome were filtered to identify potential mutations in genes previously not directly associated with SRNS.

<u>*MAGI2* mutations identified by whole exome sequencing</u>

After filtering and further analysis of potentially pathogenic mutations in genes not previously directly associated with SRNS, we identified three novel, likely disease causing frameshift mutations (Figure 1) in Membrane associated guanylate kinase, WW and PDZ domain containing 2 (*MAGI2* [MIM: 606382]) in one sporadic case (180, Figure S4) and two familial cases (175 and 175S, Figure S5) presenting with non-syndromic congenital SRNS. As parents of patient 175 and 175S were consanguineous, autosomal recessive inheritance was considered the most likely mechanism of inheritance.

Whole exome sequencing was performed on patient 175 and 180. Subsequent segregation analysis confirmed that two siblings (175 and 175S) shared the same homozygous frameshift deletion *MAGI-2*:NM_012301:exon22:c.3998delG:p.(Gly1333Alafs*141). The sporadic case (180) exhibited compound heterozygosity: a deletion (paternal) resulting in a premature stop codon *MAGI-2*:NM_012301:exon1:c.64_71delAGGAACCC:p.(Arg22Glyfs*7) together with a duplication (maternal) *MAGI-2*:NM_012301:exon20:c.3526_3533dupCTGGCAGA:p.(Glu1178Aspfs*9). All three variants were absent in ExAC database, had high CADD scores (34 (175, 175S), 35 and 35 (180)) respectively supporting pathogenicity of these mutations. Translation of the mutated protein (ExPASy http://web.expasy.org/translate) indicated that the two frameshift mutations located

in exon 1 and exon 20 resulted in stop codons and premature termination of protein translation and were located in PDZ domains. The exon 22 frameshift deletion resulted in predicted read through of the normal termination (stop) codon, resulting in likely continued translation of the mRNA further downstream into the 3'-untranslated region (UTR).

Cases 175, 175S and 180 did not demonstrate any other significant rare or novel variants in other genes expressed in podocytes during exome screening supporting *MAGI2* as the most likely causative gene. Furthermore, there were no rare frameshift mutations at the same site as any of the *MAGI2* mutations within 100bp in Ensembl or other public databases, although one in-house control carried a heterozygous frameshift insertion in exon 20:c.3512_3513insTGTA:p.(Leu1171Phefs*27). Other *MAGI2* variants were not detected in these or other control samples.

The *MAGI2* homozygous frame shift deletion p.(Gly1333Alafs*141) was verified by Sanger sequencing and this was only homozygous variant present in both 175 and 175S. It was also present in the mother (175M) as a heterozygote but could not be verified in the father as DNA was not available.

Of the eight rare/novel variants found in 175, two (*RAD51D, NUP155*) were absent in 175S and the other two (*GABRD*, *POLR2M*) were only present as heterozygous variants. Furthermore, one variant (*AKR1C1*) was present as homozygous in the ExAC database and one (*IGLL5*) was predicted to be tolerated by *in silico* tools; excluding both as likely candidates. Both heterozygous variants in *SKOR1* found in 175 were inherited from the mother and thus present on the same allele. Only one rare heterozygous potential known SRNS gene variant was shared by the siblings: *COL4A3* c.4421T>C:p.(Leu1474Pro), detected prior to enrolment to the study by the Bristol Genetics Laboratory (SRNS 37 gene panel, www.nbt.nhs.uk/severn-pathology/pathology-services/bristol-genetics-laboratory-bgl). This variant was seen over 300 times as a heterozygote in ExAC database and is therefore of unlikely significance.

Since DNA from both parents was available for 180, we were able to verify that the novel *MAGI2* mutations were in trans; p.(Arg22Glyfs*7) was inherited on a paternal allele and p.(Glu1178Aspfs*9) on a maternal allele. The frame shift variations in *MAGI2* were not present in the ExAC database. Six other nonsense variants in *MAGI2* were present; however each seen only once and as a heterozygote. Similarly one splice acceptor and two splice donor variants are also present in the ExAC database but again, only present as heterozygotes. If we include non-PASS variants an additional nonsense and 3 frameshift variants are also present, however again, all were seen only as heterozygotes.

5 other genes were also left after the filtering steps; however, variants in three genes were maternally inherited in cis, and insertions in *MICALCL* and *ZIC5* were considered of unlikely significance due to presence of similar insertions around this region in ExAC database. *MAGI2* remained the sole candidate gene for causing nephrotic syndrome in our patients. (Data is presented in Table S3)

There was no associated extra-renal phenotype in patient 175 while 175S had some minor cardiac (possibly unrelated) abnormalities. Case 180 had associated polydactyly and a previous pyloric stenosis, although did not demonstrate any other features of any characterised syndrome. The inheritance pattern in all three cases was compatible with autosomal recessive disease. All had presented with significant proteinuria and hypoalbuminaemia within the first 4 months of life compatible with a CNS. Patient 175 was diagnosed after renal biopsy with 'Finnish type' nephrotic syndrome which is usually caused by *NPHS1* mutations, but the renal histology in early life may be relatively non-specific in appearance and no *NPHS1* mutations were detected on screening the entire gene. Patient 175 had rapid disease progression and required a kidney transplant at age 3.5 years; in contrast the sibling, 175S, has followed a more benign course. Similarly patient 180 has had persistent proteinuria for 9 years with only mild renal impairment to date. Phenotypic variability between different family members is not unusual in SRNS and has been

previously described Interestingly, the most severely affected patient (175) also carried a single heterozygous variant in *LAMB2* resulting in c.4274G>C:p.(Gly1425Ala), the significance of which is unknown but was not present as a homozygote on the ExAC database and had a MAF of 0.000075. Disease modification due to bi-genic heterozygosity or or tri-allelic hits may occur, but is unusual in SRNS.[13] Phenotypic details are presented in Table 1.

Immunohistochemistry:

Kidney biopsy sections (from individual 180 and an individual with MCD), a nephrectomy section from individual 175 and from an unsuitable for transplant normal human kidney sections used for IHC were formalin fixed and paraffin embedded.

Glomeruli from patient 175 showed marked lobulation, with fibrosis or global sclerosis, interstitial fibrosis and diffuse inflammation. From patient 180, changes were milder (consistent with milder renal impairment), with 2/59 glomeruli showing global sclerosis and the rest looking normal on light microscopy. Electron microscopy (supplemental Figure S3) from this specimen shows a normal mesangial area, normal glomerular basement membrane, and diffuse podocyte foot process effacement, similar to the reported mouse model[14].

MAGI2 staining was absent in case 180, consistent with an early truncation resulting in non-translation of the MAGI2 protein through nonsense mediated decay. MAGI2 staining was however weakly positive in podocytes of case 175. The terminal position of the frame shift mutation (exon 22) and predicted read through into the 3' UTR supports weak expression of a translated yet dysfunctional protein.

We observed MAGI2 staining in the tubules in control sections, which was absent in case 180 with this antibody (Sigma). We therefore stained sections with a different antibody

(Santa Cruz) which showed different degrees of tubular staining in all sections, weak glomerular staining in the normal kidney, weak/negative staining in case 175 and negative staining in case 180 (Figure S2). The 'best fit' explanation for the difference in tubular staining with the Santa Cruz antibody is that this is due to a component of non-specific staining with this antibody, so we cannot be conclusive about the specificity of tubular staining.

Nephrin expression appeared to be decreased and localisation altered in both 175 and 180 patients compared to control, and similar to what was observed in MAGI2 mouse models.[15, 16]

Discussion

We performed whole exome sequencing using an Illumina platform on 187 SRNS cases as an extension of previous analysis of a cohort of childhood SRNS[1]. Cases lacking mutations in the known 53 nephrotic genes were analysed further in particular those with congenital nephrotic syndrome which is generally autosomal recessive and a developmental disorder. The exome data was therefore analysed to look for novel candidate genes. After filtering and further analysis of potentially pathogenic mutations in genes expressed in podocytes but not previously directly associated with SRNS, we identified likely disease causing frameshift mutations in *MAGI2* in one sporadic case (180) and two familial cases (175 and 175S).

Although a recently ascribed podocyte gene, *MAGI2* mutations have been not previously been directly associated with human SRNS. MAGI2 together with its paralogues MAGI1 and MAGI3 belong to the membrane-associated guanylate kinase (MAGUK) family of scaffolding proteins highly expressed in neurones and normally associated with neurological function [17-19]. MAGI proteins function as molecular scaffolds coordinating signalling complexes by linking cell surface receptors to the cytoskeleton but differ from other members of the MAGUK family by having a guanylate kinase (GK) domain at the N-terminus as well as WW

and PDZ domains in reverse orientation to MAGUK[20]. MAGI2 is expressed in podocyte foot processes together with IQGAP1, CASK, spectrins and α-actinin and is known to directly bind the cytosolic tail of nephrin, an essential component of the slit diaphragm, as part of the nephrin multi-protein complex[21]. It also plays a role in RhoA dependent regulation of the actin cytoskeleton, an important process in podocytes[22]. Moreover, *MAGI2* has recently been described as a WT1 target gene and required for podocyte development both in zebrafish[23] and mice. Mice with *MAGI2* deletion display disruption of the slit diaphragm formation, podocyte foot process effacement, severe glomerular pathology compatible with human SRNS, with early death from nephrotic syndrome and kidney failure[16]. In humans, expression data from micro-dissected renal biopsies (Nephromine http://www.nephromine.org/) indicated *MAGI2* was one of the top deregulated genes in glomerular disease with a 3.2-fold and 6.2-fold decrease in focal segmental glomerulosclerosis (FSGS) and diabetic nephropathy respectively, further underlining its potential importance in human SRNS.[15]

Aside from published evidence that MAGI2 is a component of the multi-protein complex at the slit diaphragm, *MAGI2* deleted mice die soon after birth from podocyte injury, severe proteinuria and end stage renal failure, indicating a profound developmental slit diaphragm defect[17,19,20,22]. Furthermore, MAGI2 is a WT1 target during podocyte development and has been previously implicated in RhoA regulation/signalling known to play a critical role in actin cytoskeletal regulation in podocytes. [23 22 24 25]

In conclusion, we present genetic data that supports *MAGI2* mutations as a cause of congenital SRNS in humans. Although further functional studies are required to establish exactly how *MAGI2* mutations lead to human CNS, we propose *MAGI2* gene mutations should be added to gene panels when investigating SRNS, and considered in patients with congenital onset SRNS, particularly where mutation in other known genes is not found.

Methods

<u>Sequencing</u>

Patients 175 and 180 underwent exome sequencing. DNA from peripheral blood was extracted Gentra Puregene Blood Kit (Qiagen). DNA libraries were prepared from 3 μg dsDNA using SureSelect Human All Exon 50Mb kit (Agilent). Samples were multiplexed (4 samples on each lane) and 100 bp paired end sequencing was performed on Illumina HiSeq system. Sequence data was aligned to the hg19 human reference genome using Novoalign (Novocraft Technologies SDN BHD, Malaysia), variants were called with SAMtools (Sequence Alignment/Map Tools)[26] and annotated via multiple passes through Annovar[27]. Initial exploration of datasets was performed using Integrative Genome Browser (https://www.broadinstitute.org/igv).

<u>Variant filtering</u>

1. Only variants with a MAF <0.01 were considered (1000 Genomes Project http://www.1000genomes.org/, NHLBI Exome Sequencing Project (ESP http://evs.gs.washington.edu/EVS/), http://exac.broadinstitute.org/, KCL in-house data set (King's College London, in-house data from 5000 control individuals without kidney disease)).

2. Variants seen as a homozygote in ExAC database, ESP or 1000 Genomes Project were excluded from further analysis.

3. For the novel/not previously described missense variants, information from Alamut Visual http://interactive-biosoftware.com/alamut-visual and UCSC http://genome.ucsc.edu/ was used to check whether an amino acid is conserved – For filtering, the amino acid must be conserved and the new amino acid must not be present in another multicellular organism.

4. Analysis of potential synonymous and splice site variants using Alamut Visual 2.7 (SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer, Human Splicing

Finder) to check if there was a consistent predicted splice effect across the majority of tools. *MAGI2* frameshift mutations were examined for deleteriousness using CADD http://cadd.gs.washington.edu.

5. Variants present within 50bp and subsequently shown to be present in cis were excluded and not considered compound heterozygotes.

6. Any variants that did not meet criteria above were discarded

7. All mutations of interest were confirmed by Sanger sequencing in probands and parents.

To ascertain further annotative information about potential genes of interest we also examined genomic, proteomic, transcriptomic, genetic and functional information on all new candidates using databases such as GeneCards http://www.genecards.org/, Ensembl http://ensembl.org   and Uniprot http://www.uniprot.org as well as the Human Protein Atlas http://www.proteinatlas.org/   and literature searches to help decisions regarding potential pathogenicity of findings. Mouse Genome Informatics (MGI http://www.informatics.jax.org/) was used for murine data and establishing whether SRNS disease phenotypes might occur in mice. Further details are provided in Table S3 and S4.

Immunohistochemistry:

Two MAGI2 antibodies were used:

MAGI2 (Sigma, #HPA013650) was used 1:250. Heat-induced epitope retrieval was performed using a domestic stainless steel pressure cooker. 5 minutes were counted as soon as the cooker has reached full pressure. (10mM Sodium citrate tribasic pH 6). 10% goat serum (Sigma) was used for blocking.

MAGI2 (Santa Cruz, #sc-25664) was used 1:50. Heat induced antigen retrieval method was used (boiled for 5 min, 10mM Sodium citrate tribasic pH 6). 3% BSA + 3% goat serum was used for blocking.

<u>Fluorescent Immunohistochemistry staining of paraffin-embedded tissue sections.</u>

Nephrin (R&D, #AF4269) was used 1:200. Donkey anti-sheep IgG (H+L) secondary antibody, Alexa Fluor® 488 conjugate (Life Technologies, #A11015) was used in 1:300. Proteinase K was used as antigen retrieval (20µg/ml). 1.5% BSA (Sigma, #A9647) plus 5% donkey serum (SIGMA, # D9663) was used for blocking.

Acknowledgments

Figures and Tables

Figure 1. MAGI2 mutations causing Congenital Nephrotic Syndrome.

A. The exon structure of *MAGI2* cDNA (NM_012301.3). 22 coding exons with start and stop codon indicated.
B. Domain structure of the MAGI2 protein. Six PDZ domains (PDZ0-PDZ5) in blue, one guanylate kinase domain (GK) in yellow and two WW domains in green.
C. Frame shift variants found in three patients with CNS. Individual 180 is a compound heterozygote, variant in exon 1 was inherited from the Father and in exon 20 from the Mother. Individual 175 and her sister, 175S, are homozygous for a single nucleotide deletion in exon 22. Mother of the siblings is heterozygous for the variant.

Patient #	Gender	Ethnicity	Familial / Sporadic	Age at onset	Consanguinity	Resistance to steroids	1st native biopsy	2nd native biopsy	CKD stage	Time to ESRF	Transplanted?	Disease recurrence?	Extra-renal phenotype	Treatment used	Most recent Serum Creatinine (umol/L)	Most recent Albumin Creatinine Ratio (mg/mmol)	Length of follow up (years)
180	M	W	S	3mo	No	presumed	MCD (28mo)	Na	2	N/A	No	N/A	Pyloric stenosis, Single digit post-axial Polydactyly, Thrombocytosis	Enalapril Cyclosporin, Tacrolimus, Prednisolone, Mycophenolate mofetil,	32	83 (serum albumin 17g/L)	9.08
175	F	W	F	4mo	Yes	presumed	Late Finnish type CNS (22mo)	End stage of Finnish type CNS (26mo)	Tx	1y9m	Yes	No	No	None	73	3.2	9.58
175S	F	W	F	1mo	Yes	presumed	Na	Na	1	Na	Na	Na	mild peripheral branch pulmonary stenosis; Patent foramen ovale	Captopril,	24	2160 (serum albumin 17g/L)	2.33

Table 1. Clinical features of the affected individuals with MAGI2 mutations.

The following abbreviations are used: M – male, F – female; mo – months; MCD – Minimal Change Disease, CNS – Congenital Nephrotic Syndrome; CKD – Chronic Kidney Disease; ESRF – End Stage Renal Failure; Tx – transplanted;

Figure 2. MAGI2 staining.

Immunohistochemical staining with human anti-MAGI2 antibody (Sigma) is shown (20x). A - A kidney which was not suitable for transplant was used as a control, B - Biopsy specimen obtained from an individual with Minimal Change Disease, MAGI2 staining is seen in the glomeruli at the periphery of capillary loops, consistent with podocyte localisation. Staining in tubules is also seen. C - Nephrectomy specimen obtained from 175 (homozygous mutation in MAGI2) shows weak but positive glomerular MAGI2 staining D - Biopsy specimen obtained from 180 (compound heterozygous mutation in *MAGI2*) shows complete lack of MAGI2. Black line indicates 100µm scale.

Figure 3. Nephrin immunostaining on paraffin embedded renal tissue sections.

Confocal microscopy images shows nephrin staining (green), DAPI nuclear staining in blue. A – positive control, normal human kidney; B – negative control (no nephrin antibody), normal human kidney; C - Nephrectomy specimen obtained from 175 (homozygous mutation in *MAGI2*); D - Biopsy specimen obtained from 180 (compound heterozygous mutation in *MAGI2*). Top panel shows whole glomerulus (40x +/-3), bottom panel A, C, D, shows higher magnification (100x +/-7). Bottom panel shows decreased/disrupted nephrin expression between normal and patients' podocytes.

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